PH.D. THESIS

GENETIC MODIFICATION OF CAROTENE PRODUCING ZYGOMYCETES

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Introduction

Zygomycetes fungi have great practical importance in medical, industrial, biotechnological and agricultural aspects. Some species are considered as opportunistic pathogens causing zygomycosis, others are known as model-organisms in genetic studies and several species are used in the biotechnological industry.

Carotenoids are one of the most important of natural pigments with a broad range of biological functions. They play an important role in photosynthetic and heterotrophic organisms. Their effective antioxidant property linked to a preventive action on various types of cancer and an enhancement of the immune response makes them important in the human diet. Carotenoids are used in the food, pharmaceutical and cosmetic industries and as feed colour additives. Today most of the carotenoids are produced by chemical methods, but there is an increasing interest for sources of carotenoids of biological, especially of microbiological origins. Carotenoid productivity in low amount is the major obstacle to the usage of carotenoids from microbiological source. The main industrial carotenoid is β-carotene and it is also the main product of the synthesised carotenoids in Zygomycetes. Today, both applied and basic researches are increasingly directed towards the oxygenated derivatives of the β-carotene (xanthophylls), especially to the biosynthesis of the red keto-derivatives.

Zygomycetes, especially the members of the genera *Phycomyces, Blakeslea* and *Mucor* have been used as model-organism for the study of the genetic and biochemical background of the carotenoid biosynthesis. In the *Mucor* genus, the carotenoid biosynthesis of the *Mucor circinelloides* is the most studied. This fungus has some advantageous features in reference to molecular studies and biotechnological applications, such as the possibility of the genetic transformation, the expression of heterologous genes or the morphological dimorphism. Mutant strains, with

changed carotenoid production were also isolated. Many genes, in connection with the carotenoid biosynthesis pathway have been cloned and characterized.

Transformation systems resulting stable transformants are necessary for the functional analysis of the genes and the genetic modification of the biotechnologically important organisms. At the same time the numbers of the Zygomycetes species, which have been successfully transformed, are low. Some properties of Zygomycetes make the genetic analysis or modifications difficult, like the coenocytic mycelial structure; a genomic defence mechanism, which eliminate the foreign DNA; and very poor known about the fate of introduced DNA has. If circular plasmids are used for genetic transformation, Zygomycetes fungi commonly maintain the introduced plasmids as autonomously replicating elements without any integration event. Integration can achieve, even if the introduced vector carries homologous regions directing the recombination with the genomic DNA. Recently the *Agrobacterium tumefaciens*-mediated transformation was also achieved at fungi, which also resulting integration to the host genome.

Aims

Our aim was the modification of the carotenoid production of *M. circinelloides* to increase the β-carotene production and to synthesise other important carotenoids (e.g. canthaxanthin and astaxanthin) using recombination and genetic transformation techniques. We also planned the testing of different methods, which could be useful in the further research for analysis and characterization of the genetic transformation methods and the isolated transformants.

Taking into account all these aspects, we set the following objectives:

1. Production of xanthophylls by genetic modification of the carotenoid producer *M. circinelloides* double auxotrophic mutant MS12 strain. Autonomously replicating plasmids harbouring the astaxanthin biosynthesis genes of the marine bacterium, *Paracoccus* sp. N81106 are planned to be used in the experiments.

2. Cloning and characterization of the *M. circinelloides* isopentenyl pyrophosphate isomerase coding gene. Increasing of the β-carotene production of the *M. circinelloides* by overexpression of three non-carotenoid specific genes of the terpene biosynthesis (i.e. *ipi*, *isoA* and *carG*), using autonomously replicating plasmids.

3. Xanthophyll production by integration of bacterial genes into the *M. circinelloides* genome, using different integrative transformation methods.

Methods

DNA and RNA based techniques:

- ✓ DNA extraction
- ✓ RNA extraction and cDNA synthesis
- ✓ DNA/RNA gel electrophoresis
- ✓ Polymerase chain reaction, inverse-PCR, real-time PCR (qPCR)
- ✓ Cloning of DNA fragments, DNA sequencing
- ✓ Plasmid construction
- ✓ Transformation of bacteria
- ✓ Plasmid DNA extraction
- ✓ Plasmid rescue
- ✓ Southern hybridization
- ✓ Northern hybridization

Analysis of the nucleotide and the amino acid sequence data:

- ✓ Analysis and comparison of the nucleotide sequences
- ✓ Deduction of the amino acid sequences from nucleotide sequences
- ✓ Alignment of the nucleotide and the amino acid sequences

Genetic transformation of fungi:

- ✓ Generation of fungal protoplasts
- ✓ PEG-mediated protoplast transformation using autonomously replicating plasmids and vectors for the gene integration into the fungal genome
- ✓ *Agrobacterium tumefaciens*-mediated transformation
- ✓ Generation of monosporangial clones, analysis of mitotic stability

Analytical methods

- ✓ Fungal carotenoid extraction
- ✓ Analysis of the carotenoid samples using spectrophotometry, Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC) techniques.

Results

1. Heterologous expression of the *Paracoccus* sp. N81106 strain *crtZ* (β-carotene hydroxylase) and *crtW* (β-carotene ketolase) genes in *M. circinelloides*.

The *Paracoccus* sp. N81106 strain *crtZ* and *crtW* genes were placed under the control of the *M. circinelloides gpd1* (encoded the glyceraldehyde-3-phosphate dehydrogenase) regulatory regions. Transformation and co-transformation experiments with the two genes were also performed. The expression of the heterologous genes in the fungus was verified by Northern hybridization.

Production of zeaxanthin and β-cryptoxanthin (the hydroxy derivatives of β-carotene) in the wild type *M. circinelloides* was demonstrated, so the fungus has β-carotene hydroxylase activity. Therefore, expression of the *crtW* gene encoding the β-carotene ketolase seems to be enough to produce canthaxanthin, echinenone, astaxanthin, and other ketolated β-carotene derivatives. Changes in the carotenoid composition of the transformants harbouring the *crtW* gene were analysed in comparison with the wild type strain; production of canthaxanthin, echinenone and low amounts of astaxanthin was verified. Production of γ-carotene in the wild type *M. circinelloides* was also verified.

2. Overexpression of the isoprenoid biosynthetic genes in *M. circinelloides,* analysis of the carotenoid production of the transformants and the fate of the introduced DNA.

In Zygomycetes, the carotenoids are synthesised by a side route of the general mevalonate – isoprenoid biosynthetic pathway. We would like to answer the questions, that overexpression of the isoprenoid biosynthetic pathway genes in *M. circinelloides* had a positive effect or not to the total carotenoid production, how much is the increase and which overexpressed gene results in the highest carotenoid content.

The *ipi* gene of *M. circinelloides*, encoding the isopentenyl pyrophosphate isomerase was cloned and characterized. The identified DNA sequence contains the 910 bp long open reading frame (ORF). An 787 bp upstream and an 361 bp downstream regions also included. The whole nucleotide and the deduced amino acid sequences were deposited in the EMBL nucleotide sequence database (EMBL accession number: AM903092). The gene contains four introns (57, 57, 61 and 57 bp) and encodes a 225 amino acid long protein. The molecular weight of the predicted IPP isomerase is 26.124 kDa; the catalic region (18 – 208 AA) contains the conserved NUDIX motif of hydrolase domains (NUcleoside DIphosphate linked to some other moiety X, 49 - 199 AA). The cystein and glutamic acid residues (Cys85, C^{85} és Glu147, E^{147}), which are important in the characterization of the active site were also identified.

The *M. circinelloides isoA* and *carG* genes, encoding the farnesyl pyrophosphate synthase and the geranylgeranyl pyrophosphate synthase were isolated and characterized by Velayos et al. (2003, 2004). Autonomously replicating plasmids harbouring the *isoA*, *carG* and the *ipi* genes were constructed. The three isoprenoid biosynthesis genes were also placed under the control of promoter and terminal regions of the *M. circinelloides gpd1* gene. This promoter is very effective and its activity

depends on the concentration of glucose. The plasmid harbouring the bacterial *crtW* gene under the control of the *Mucor gpd1* regulatory regions was also involved in the transformation experiments. Transformations and co-transformations with two plasmids in all possible gene combinations were performed with PEG-mediated protoplast transformation.

Southern hybridization experiments verified that the transformants maintain the introduced DNA as autonomously replicating elements. Plasmid rearranges were detected in several transformants. The qPCR analysis verified that the *ipi*, *isoA* and *carG* genes have a single copy in the wild type *Mucor* genome. The copy number of the plasmids in the transformants was also analysed. It varied between 0.07-7 copy/genome and fluctuation in the copy numbers during the consecutive cultivation cycles was detected. The copy number of the two plasmids was often different in the co-transformants. In the same transformant, the copy number of the plasmid harbouring the heterologous gene was lower than that of the plasmid carrying one of the homologous genes. All of the analysed transformants seemed to be stable under selective and non-selective conditions too.

The transcription level of the isoprenoid pathway genes was measured comparing them with those of the actin genes and each other. The time and glucose concentration dependence of their transcription were also determined with qPCR technique. All the three genes have a low transcription level in comparison with actin. In comparison with *carG*, *ipi* and *isoA* showed higher transcription levels at only 4 h after inoculation indicating that the transcription of these genes is active in the germinating spores. After germination, their expression decreased and stabilized at a relatively low level. However, the transcription level of *ipi* remained about twice of those of the other two genes, even on the fourth day of cultivation. The expression level of *carG* proved to be relatively low throughout the

cultivation period. In the case of the untransformed MS12 strain, the transcription of all three genes underwent a slight decrease with increasing glucose concentration. The transcription levels of the introduced genes were measured and their increases were verified. The expression of the bacterial gene was also verified. In the transformants, the transcription of the genes was significantly increased at 2.5% glucose as compared with that at 1%. At 5% glucose, it was similar to or slightly lower than the level at 2.5%.

After optimization of the parameters, spectrophotometry, TLC and HPLC techniques were used for the analysis of the carotenoid production. The fluctuation in the copy number of the plasmids and in the relative transcription levels resulted in fluctuating carotenoid production as well. Overexpression of the isoprenoid pathway genes increased the carotenoid production. Overexpresssion of the *carG* gene resulted in the highest carotenoid accumulation. Some of the co-transformants produced three or four times more carotenoids, than the wild type strain. No significant changes were detected in the composition of the produced carotenoid species in the transformants in comparison with the wild type strain. Introduction of the bacterial gene, the *carG+crtW* and *ipi+crtW* gene combinations resulted the highest carotenoid production and also the highest concentration of the β-carotene keto-derivatives, mainly canthaxanthin and echinenone. The concentration of astaxanthin was low in all of the analysed transformants. The total carotenoid contents detected at 2.5% and 5% glucose were found to be lower than those measured at 1%, if the carotenoid content was referred to the dry mass of the mycelia, however the biomass increased.

3. Integration of the *crtW* gene into the *M. circinelloides* genome using different methods.

Previously it has been shown that the transformants maintain the plasmids as autonomously replicating elements without any integration

event. Because of the fluctuation in the copy number of the plasmids, in the transcription levels of the introduced genes and in the carotenoid production of the transformants as well, the integration of the introduced DNA would be necessary. The integration of the bacterial *crtW* gene into the *M. circinelloides* genome was achieved by different methods: gene substitution based on double crossing over; restriction enzyme-mediated integration (REMI) and *Agrobacterium tumefaciens*-mediated transformation (ATMT). After the optimization of the transformation conditions, successful transformations were performed with all of the constructed vectors. With the exception of the ATMT method, stable transformants were isolated with each method.

PCR technique was used to verify the presence of the *crtW* gene in the transformants. Southern hybridization technique was used to verify the integration and inverse-PCR to determine the site of the integration. The integration into the *Mucor* genome was often in one copy, at the same time the REMI and ATMT techniques resulted multi copy integrations as well. DNA rearranges were also detected. The integration by substitution was verified in one case, but most of the methods resulted ectopic integration. Southern hybridizations revealed possible hot-spots, but the sequences could not be identified.

The copy number of the bacterial gene was analysed with qPCR. In some of the transformants it was very low varying between 0.001-0.5 copy/genome. In some of these transformants, the copy number of the gene increased with the cultivation cycles to around one copy per host genome reaching the homokaryotic state. In others, the heterokaryotic state was permanent and copy number not increased significantly. In few of the transformants, the copy number of the *crtW* gene was high directly after the transformation; and after more than 13 cultivation cycles, it reached more than 200 copies per genome. Based on the Southern hybridization and

inverse-PCR analyses, this extremely high copy number can be explicated by the excision, circularization and amplification of the integrated DNA. Maybe the introduced linear fragments ligated to each other forming large concatamer structures. This molecule could also be integrated into the genome resulting several tandem repeats. Expression of the bacterial gene in the transformants was verified, the relative transcription levels increased with the increasing copy number of the transferred *crtW* gene.

Carotenoid production of the transformants was analysed and several mutant strains producing xanthophylls in high amounts (mainly canthaxanthin and echinenone) were isolated. In such strains, the *crtW* gene was detected in extremely high copy number. These strains were cultured under different conditions, such as different temperatures, light sources and media, which contained different carbon sources or chemical additives. The lower temperature resulted in higher concentration of β-carotene keto derivatives. Several culture conditions were determined for these strains where the β-carotene – canthaxanthin conversion was nearly complete. Production of astaxanthin was near to zero in these strains. Maybe the β-carotene hydroxylase of *M. circinelloides* is able to use the canthaxanthin as substrate only with very low efficiency. For this reason, the autonomously replicating plasmid carrying the *crtZ* gene was used for the transformation of two of the canthaxanthin-producing integrative mutants. As a result, astaxanthin production in high amount was detected in these strains.

A plasmid carrying the *crtW-carRP* gene fusion was also built and used for the transformation of the MS12 strain, but these transformants produced low amount of carotenoids, most likely because the fused protein inhibited the *Mucor* CarRP activity.

Summary

1. Autonomously replicating plasmids harbouring the *Paracoccus* sp. N81106 strain *crtZ* and *crtW* genes were constructed. Transformations and co-transformation of the *M. circinelloides* MS12 strain with the plasmids were successful. Co-transformation of *M. circinelloides* was firstly achieved by our research group.
2. Transformation frequencies was analysed and expression of the heterologous genes in the MS12 were verified.
3. The *M. circinelloides* β-carotene hydroxylase activity was verified.
4. Production of ketolated β-carotene derivatives in the transformants harbouring the *crtW* gene was demonstrated.
5. γ-Carotene production of *M. circinelloides* was also verified.
6. The *ipi* gene, encoding the isopentenyl pyrophosphate isomerase of *M. circinelloides* was cloned and characterized.
7. It was proven that the *ipi*, *isoA* and *carG* genes have the same one copy number in the *Mucor* genome. Transcription level of these isoprenoid genes was analysed.
8. Expression vectors harbouring the *M. circinelloides ipi*, *isoA* and, *carG* genes were constructed. The genes were placed under the control of the *Mucor gpd1* regulatory regions. PEG-mediated transformations and co-transformations of the MS12 protoplasts were performed with all possible gene-combinations. The plasmid harbouring the bacterial *crtW* gene was also involved in the transformation experiments.
9. Transformation frequencies were analysed. The transformants maintained the plasmids as autonomously replicating extrachromosomal elements. DNA rearrenges were detected.
10. Stability of the transformants, copy number of the introduced plasmids and transcription levels of the introduced genes under different culturing conditions were also analysed.
11. Effect of different culturing conditions on the carotenoid production was tested.

12. Vectors for integration of the *crtW* gene into the *Mucor* genome were constructed and the integration was successfully performed with different transformation methods.
13. The transformation frequencies were analysed. Southern hybridization and inverse-PCR technique were used to verify the integration. DNA rearranges were detected.
14. Relative copy number of the *crtW* gene and changes in the transcription level of the gene during the consecutive cultivation cycles were analysed.
15. Carotenoid production of the transformants were analysed; accumulation of the keto-derivatives of β-carotene was shown. Carotenoid production under different culturing conditions was tested as well.
16. The bacterial *crtZ* gene was introduced into these transformants, using autonomously replicating plasmids. Increased astaxanthin production could be achieved using this method.
17. An autonomously replicating vector, harbouring the *carRP*-*crtW* gene fusion was constructed and the MS12 strain was transformed with it. The copy number of the introduced DNA and the carotenoid production of the transformants were analysed.

The results summarized in the Ph. D. thesis were published in the following articles:

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